

09/830446

PCT / CA 99/00992

24 FEBRUARY 2000 (24.02.00)

CA 99/992

REC'D 08 MAR 2000

WIPO PCT

PA 201862

4

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

February 10, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/106,087

FILING DATE: October 29, 1998

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)



By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

A. L. Jackson
H. L. JACKSON
Certifying Officer

Please type a plus sign (+) inside this box → ☒

PTO/SB/16 (12-87)
Approved for use through 1/31/98. OMB 0651-0037
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)		Residence
Given Name (first and middle, if any)	Family Name or Surname	(City and either State or Foreign Country)
Andrew D	Murdina	146 Rhodes Circle, Newmarket, Ontario, Canada L3Y 1V2
Raymond P	Oomen	RR No 1 Schomberg, Ontario, Canada
<input type="checkbox"/> Additional inventors are being named on the <u>LOG-110</u> separately numbered sheets attached hereto.		
TITLE OF THE INVENTION (280 characters max)		
Chlamydia Antigens and Corresponding DNA Fragments and Uses Thereof		
CORRESPONDENCE ADDRESS		
Direct all correspondence to:		Place Customer Number Bar Code Label here
<input type="checkbox"/> Customer Number <input type="text"/> →		
OR Type Customer Number here		
<input checked="" type="checkbox"/> Firm or Individual Name	Gavin R. Zealey	
Address	1755 Steeles Ave West	
Address		
City	Toronto	State Ontario ZIP M2R 3T4
Country	Canada	Telephone 416-667-2854 Fax 416-667-2860
ENCLOSED APPLICATION PARTS (check all that apply)		
<input checked="" type="checkbox"/> Specification Number of Pages	31	<input type="checkbox"/> Small Entity Statement
<input checked="" type="checkbox"/> Drawing(s) Number of Sheets	13	<input type="checkbox"/> Other (specify) <input type="text"/>
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)		
<input type="checkbox"/> A check or money order is enclosed to cover the filing fees		FILING FEE AMOUNT (\$)
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 50-0244		150.00
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.		
<input checked="" type="checkbox"/> No.		
<input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: _____		

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME Gavin R. Zealey

TELEPHONE 416-667-2854

Date

10/27/98

REGISTRATION NO.

39,475

(if appropriate)

Docket Number

RY-32

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231.

TITLE OF INVENTION

**CHLAMYDIA ANTIGENS AND CORRESPONDING DNA FRAGMENTS AND USES
THEREOF**

FIELD OF INVENTION

5 The present invention relates to *Chlamydia* antigens and corresponding DNA molecules, which can be used in methods to prevent and treat *Chlamydia* infection in mammals, such as humans.

BACKGROUND OF THE INVENTION

10 Chlamydiae are prokaryotes. They exhibit morphologic and structural similarities to gram-negative bacteria including a trilaminar outer membrane, which contains lipopolysaccharide and several membrane proteins that are structurally and functionally analogous to proteins found in *E coli*. They are obligate intra-cellular parasites with a unique biphasic life cycle consisting of a metabolically inactive but infectious extracellular stage and a replicating but non-infectious intracellular stage. The replicative stage of the life-cycle takes place within a membrane-bound inclusion which sequesters the bacteria away from the cytoplasm of the infected host cell.

15 *C. pneumoniae* is a common human pathogen, originally described as the TWAR strain of *Chlamydia psittaci* but subsequently recognised to be a new species. *C. pneumoniae* is antigenically, genetically and morphologically distinct from other chlamydia species (*C. trachomatis*, *C. pecorum* and *C. psittaci*). It shows 10% or less DNA sequence homology with either of *C. trachomatis* or *C. psittaci* and so far appears to consist of only a single strain, TWAR.

20 *C. pneumoniae* is a common cause of community acquired pneumonia, only less frequent than *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* (Ref 1,2). It can also cause upper respiratory tract symptoms and disease, including bronchitis and sinusitis (Ref 1,3,4,5). The great majority of the adult population (over 60%) has antibodies to *C. pneumoniae* (Ref 5), indicating past infection which was unrecognized or asymptomatic.

25 Of considerable importance is the association of atherosclerosis and *C. pneumoniae* infection. There are several epidemiological studies showing a correlation of previous infections with *C. pneumoniae* and heart attacks, coronary artery and carotid artery disease (Ref 6-10). Moreover, the organisms has been detected in atheromas and fatty streaks of the coronary, carotid, peripheral arteries and aorta (Ref 11-15). Viable *C. pneumoniae* has been recovered

from the coronary and carotid artery (Ref 16,17). Furthermore, it has been shown that *C. pneumoniae* can induce changes of atherosclerosis in a rabbit model (Ref 18). Taken together, these results indicate that it is highly probable that *C. pneumoniae* can cause atherosclerosis in humans, though the epidemiological importance of chlamydial atherosclerosis remains to be demonstrated.

A number of recent studies have also indicated an association between *C. pneumoniae* infection and asthma. Infection has been linked to wheezing, asthmatic bronchitis, adult-onset asthma and acute exacerbations of asthma in adults, and small-scale studies have shown that prolonged antibiotic treatment was effective at greatly reducing the severity of the disease in some individuals (Ref 19-24).

In light of these results a protective vaccine against *C. pneumoniae* infection would be of considerable importance. There is not yet an effective vaccine for any human chlamydial infection. Nevertheless, studies with *C. trachomatis* and *C. psittaci* indicate that this is an attainable goal. For example, mice which have recovered from a lung infection with *C. trachomatis* are protected from infertility induced by a subsequent vaginal challenge (Ref 25). Similarly, sheep immunized with inactivated *C. psittaci* were protected from subsequent chlamydial-induced abortions and stillbirths (Ref 26). Protection from chlamydial infections has been associated with Th1 immune responses, particularly the induction of INF γ -producing CD4⁺T-cells (Ref 27). The adoptive transfer of CD4⁺ cell lines or clones to nude or SCID mice conferred protection from challenge or cleared chronic disease (Ref 28,29), and in vivo depletion of CD4⁺T cells exacerbated disease post-challenge (Ref 30,31). However, the presence of sufficiently high titres of neutralising antibody at mucosal surfaces can also exert a protective effect (Ref 32).

The extent of antigenic variation within the species *C. pneumoniae* is not well characterised. Serovars of *C. trachomatis* are defined on the basis of antigenic variation in MOMP, but published *C. pneumoniae* MOMP gene sequences show no variation between several diverse isolates of the organism (Ref 33-35). Regions of the protein known to be conserved in other chlamydial MOMPs are conserved in *C. pneumoniae* (Ref 33,34). One study has described a strain of *C. pneumoniae* with a MOMP of greater than usual molecular weight, but the gene for this has not been sequenced (Ref 1). Partial sequences of outer membrane protein 2 from nine diverse isolates were also found to be invariant (Ref 16). The genes for

HSP60 and HSP70 show little variation from other chlamydial species, as would be expected. The gene encoding a 76kDa antigen has been cloned from a single strain of *C. pneumoniae*. It has no significant similarity with other known chlamydial genes (Ref 4).

Many antigens recognised by immune sera to *C. pneumoniae* are conserved across all chlamydiae, but 98kDa, 76 kDa and 54 kDa proteins may be *C. pneumoniae*-specific (Ref 2, 4, 36). Immunoblotting of isolates with sera from patients does show variation of blotting patterns between isolates, indicating that serotypes *C. pneumoniae* may exist (Ref 1,16). However, the results are potentially confounded by the infection status of the patients, since immunoblot profiles of a patient's sera change with time post-infection. An assessment of the number and relative frequency of any serotypes, and the defining antigens, is not yet possible.

C. pneumoniae infection usually presents as an acute respiratory disease (i.e., cough, sore throat, hoarseness, and fever; abnormal chest sounds on auscultation). For most patients, the cough persists for 2 to 6 weeks, and recovery is slow. In approximately 10% of these cases, upper respiratory tract infection is followed by bronchitis or pneumonia. Furthermore, during a *C. pneumoniae* epidemic, subsequent co-infection with pneumococcus has been noted in about half of these pneumonia patients, particularly in the infirm and the elderly. As noted above, there is more and more evidence that *C. pneumoniae* infection is also linked to diseases other than respiratory infections.

The reservoir for the organism is presumably people. In contrast to *C. psittaci* infections, there is no known bird or animal reservoir. Transmission has not been clearly defined. It may result from direct contact with secretions, from fomites, or from airborne spread. There is a long incubation period, which may last for many months. Based on analysis of epidemics, *C. pneumoniae* appears to spread slowly through a population (case-to-case interval averaging 30 days) because infected persons are inefficient transmitters of the organism. Susceptibility to *C. pneumoniae* is universal. Reinfections occur during adulthood, following the primary infection as a child. *C. pneumoniae* appears to be an endemic disease throughout the world, noteworthy for superimposed intervals of increased incidence (epidemics) that persist for 2 to 3 years. *C. trachomatis* infection does not confer cross-immunity to *C. pneumoniae*. Infections are easily treated with oral antibiotics, tetracycline or erythromycin (2 g/d, for at least 10 to 14 d). A recently developed drug, azithromycin, is highly effective as a single-dose therapy against chlamydial infections.

In most instances, *C. pneumoniae* infection is often mild and without complications, and up to 90% of infections are subacute or unrecognized. Among children in industrialized countries, infections have been thought to be rare up to the age of 5 y, although a recent study (E Normann et al, *Chlamydia pneumoniae* in children with acute respiratory tract infections, *Acta Paediatrica*, 1998, Vol 87, Iss 1, pp 23-27) has reported that many children in this age group show PCR evidence of infection despite being seronegative, and estimates a prevalence of 17-19% in 2-4 y olds. In developing countries, the seroprevalence of *C. pneumoniae* antibodies among young children is elevated, and there are suspicions that *C. pneumoniae* may be an important cause of acute lower respiratory tract disease and mortality for infants and children in tropical regions of the world.

From seroprevalence studies and studies of local epidemics, the initial *C. pneumoniae* infection usually happens between the ages of 5 and 20 y. In the USA, for example, there are estimated to be 30,000 cases of childhood pneumonia each year caused by *C. pneumoniae*. Infections may cluster among groups of children or young adults (e.g., school pupils or military conscripts).

C. pneumoniae causes 10 to 25% of community-acquired lower respiratory tract infections (as reported from Sweden, Italy, Finland, and the USA). During an epidemic, *C. pneumoniae* infection may account for 50 to 60% of the cases of pneumonia. During these periods, also, more episodes of mixed infections with *S. pneumoniae* have been reported.

Reinfection during adulthood is common; the clinical presentation tends to be milder. Based on population seroprevalence studies, there tends to be increased exposure with age, which is particularly evident among men. Some investigators have speculated that a persistent, asymptomatic *C. pneumoniae* infection state is common.

In adults of middle age or older, *C. pneumoniae* infection may progress to chronic bronchitis and sinusitis. A study in the USA revealed that the incidence of pneumonia caused by *C. pneumoniae* in persons younger than 60 years is 1 case per 1,000 persons per year; but in the elderly, the disease incidence rose three-fold. *C. pneumoniae* infection rarely leads to hospitalization, except in patients with an underlying illness.

SUMMARY OF THE INVENTION

The present invention provides purified and isolated DNA molecules that encode *Chlamydia* polypeptides designated CPN100397 (SEQ ID No: 1,2), which can be used in

methods to prevent, treat, and diagnose *Chlamydia* infection. The encoded polypeptides include polypeptides having the amino acid sequence shown in SEQ ID No:3 and 4. Those skilled in the art will appreciate that the invention also includes DNA molecules that encode mutants and derivatives of such polypeptides, which result from the addition, deletion, or substitution of non-essential amino acids as described herein. The invention also includes RNA molecules corresponding to the DNA molecules of the invention.

In addition to the DNA and RNA molecules, the invention includes the corresponding polypeptides and monospecific antibodies that specifically bind to such polypeptides.

The present invention has wide application and includes expression cassettes, vectors, and cells transformed or transfected with the polynucleotides of the invention. Accordingly, the present invention provides (i) a method for producing a polypeptide of the invention in a recombinant host system and related expression cassettes, vectors, and transformed or transfected cells; (ii) a live vaccine vector, such as a pox virus, *Salmonella typhimurium*, or *Vibrio cholerae* vector, containing a polynucleotide of the invention, such vaccine vectors being useful for, e.g., preventing and treating *Chlamydia* infection, in combination with a diluent or carrier, and related pharmaceutical compositions and associated therapeutic and/or prophylactic methods; (iii) a therapeutic and/or prophylactic method involving administration of an RNA or DNA molecule of the invention, either in a naked form or formulated with a delivery vehicle, a polypeptide or combination of polypeptides, or a monospecific antibody of the invention, and related pharmaceutical compositions; (iv) a method for diagnosing the presence of *Chlamydia* in a biological sample, which can involve the use of a DNA or RNA molecule, a monospecific antibody, or a polypeptide of the invention; and (v) a method for purifying a polypeptide of the invention by antibody-based affinity chromatography.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1 shows the nucleotide sequence of the CPN100397 (SEQ ID No: 1 – entire sequence and SEQ ID No: 2 – coding sequence) and the deduced amino acid sequence of the CPN100397 protein from *Chlamydia pneumoniae* (SEQ ID No: 3 and 4).

Figure 2 shows the restriction enzyme analysis of the gene encoding the *C. pneumoniae* CPN100397 gene.

DETAILED DESCRIPTION OF INVENTION

In the *C. pneumoniae* genome, open reading frames (ORFs) encoding chlamydial polypeptides have been identified. These polypeptides include polypeptides permanently found in the bacterial membrane structure, polypeptides that are present in the external vicinity of the bacterial membrane, include polypeptides permanently found in the inclusion membrane structure, polypeptides that are present in the external vicinity of the inclusion membrane, and polypeptides that are released into the cytoplasm of the infected cells. These polypeptides can be used in vaccination methods for preventing and treating *Chlamydia* infection.

According to a first aspect of the invention, there are provided isolated polynucleotides encoding the precursor and mature forms of *Chlamydia* polypeptides.

An isolated polynucleotide of the invention encodes (i) a polypeptide having an amino acid sequence that is homologous to a *Chlamydia* amino acid, the *Chlamydia* amino acid sequence being selected from the group consisting of:

(a) the amino acid sequences as shown: (SEQ ID No: 3 and 4)

The term "isolated polynucleotide" is defined as a polynucleotide removed from the environment in which it naturally occurs. For example, a naturally occurring DNA molecule present in the genome of a living bacteria or as part of a gene bank is not isolated, but the same molecule separated from the remaining part of the bacterial genome, as a result of, e.g., a cloning event (amplification), is isolated. Typically, an isolated DNA molecule is free from DNA regions (e.g., coding regions) with which it is immediately contiguous at the 5' or 3' end, in the naturally occurring genome. Such isolated polynucleotides could be part of a vector or a composition and still be isolated in that such a vector or composition is not part of its natural environment.

A polynucleotide of the invention can be in the form of RNA or DNA (e.g., cDNA, genomic DNA, or synthetic DNA), or modifications or combinations thereof. The DNA can be double-stranded or single-stranded, and, if single-stranded, can be the coding strand or the non-coding (anti-sense) strand. The sequence that encodes a polypeptide of the invention as shown in SEQ ID NOs: 1 and 2, can be (a) the coding sequence as shown in SEQ ID NOs: 2, (b) a ribonucleotide sequence derived by transcription of (a), or (c) a different coding sequence; this latter, as a result of the redundancy or degeneracy of the genetic code, encodes the same

polypeptides as the DNA molecules of which the nucleotide sequences are illustrated in SEQ ID NOs:1 to 2.

By "polypeptide" or "protein" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). Both terms are used interchangeably in the present application.

By "homologous amino acid sequence" is meant an amino acid sequence that differs from an amino acid sequence shown in SEQ ID No: 3 or 4, only by one or more conservative amino acid substitutions, or by one or more non-conservative amino acid substitutions, deletions, or additions located at positions at which they do not destroy the specific antigenicity of the polypeptide.

Preferably, such a sequence is at least 75%, more preferably 80%, and most preferably 90% identical to an amino acid sequence shown in SEQ ID No: 3 or 4.

Homologous amino acid sequences include sequences that are identical or substantially identical to an amino acid sequence as shown in SEQ ID No:3 or 4. By "amino acid sequence substantially identical" is meant a sequence that is at least 90%, preferably 95%, more preferably 97%, and most preferably 99% identical to an amino acid sequence of reference and that preferably differs from the sequence of reference, if at all, by a majority of conservative amino acid substitutions.

Conservative amino acid substitutions typically include substitutions among amino acids of the same class. These classes include, for example, amino acids having uncharged polar side chains, such as asparagine, glutamine, serine, threonine, and tyrosine; amino acids having basic side chains, such as lysine, arginine, and histidine; amino acids having acidic side chains, such as aspartic acid and glutamic acid; and amino acids having nonpolar side chains, such as glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, and cysteine.

Homology is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Similar amino acid sequences are aligned to obtain the maximum degree of homology (i.e., identity). To this end, it may be necessary to artificially introduce gaps into the sequence. Once the optimal alignment has been set up, the degree of homology (i.e., identity) is established by recording all of the

positions in which the amino acids of both sequences are identical, relative to the total number of positions.

Homologous polynucleotide sequences are defined in a similar way. Preferably, a homologous sequence is one that is at least 45%, more preferably 60%, and most preferably 85% identical to (i) a coding sequence of SEQ ID NOs: 1 and 2.

Polypeptides having a sequence homologous to one of the sequences shown in SEQ ID NO: 3 or 4, include naturally occurring allelic variants, as well as mutants or any other non-naturally occurring variants that are analogous in terms of antigenicity, to a polypeptide having a sequence as shown in SEQ ID NO: 3 or 4.

As is known in the art, an allelic variant is an alternate form of a polypeptide that is characterized as having a substitution, deletion, or addition of one or more amino acids that does not alter the biological function of the polypeptide. By "biological function" is meant the function of the polypeptide in the cells in which it naturally occurs, even if the function is not necessary for the growth or survival of the cells. For example, the biological function of a porin is to allow the entry into cells of compounds present in the extracellular medium. The biological function is distinct from the antigenic function. A polypeptide can have more than one biological function.

Allelic variants are very common in nature. For example, a bacterial species, e.g., *C. pneumoniae*, is usually represented by a variety of strains that differ from each other by minor allelic variations. Indeed, a polypeptide that fulfills the same biological function in different strains can have an amino acid sequence that is not identical in each of the strains. Such an allelic variation may be equally reflected at the polynucleotide level.

Support for the use of allelic variants of polypeptide antigens comes from, e.g., studies of the *Chlamydial* MOMP antigen. The amino acid sequence of the MOMP varies from strain to strain, yet cross-strain antibody binding plus neutralization of infectivity occurs, indicating that the MOMP, when used as an immunogen, is tolerant of amino acid variations.

Polynucleotides, e.g., DNA molecules, encoding allelic variants can easily be retrieved by polymerase chain reaction (PCR) amplification of genomic bacterial DNA extracted by conventional methods. This involves the use of synthetic oligonucleotide primers matching upstream and downstream of the 5' and 3' ends of the encoding domain. Suitable primers can be designed according to the nucleotide sequence information provided in SEQ ID NOs: 1 and 2.

Typically, a primer can consist of 10 to 40, preferably 15 to 25 nucleotides. It may be also advantageous to select primers containing C and G nucleotides in a proportion sufficient to ensure efficient hybridization; e.g., an amount of C and G nucleotides of at least 40%, preferably 50% of the total nucleotide amount.

Useful homologs that do not naturally occur can be designed using known methods for identifying regions of an antigen that are likely to be tolerant of amino acid sequence changes and/or deletions. For example, sequences of the antigen from different species can be compared to identify conserved sequences.

Polypeptide derivatives that are encoded by polynucleotides of the invention include, e.g., fragments, polypeptides having large internal deletions derived from full-length polypeptides, and fusion proteins.

Polypeptide fragments of the invention can be derived from a polypeptide having a sequence homologous to any of the sequences shown in SEQ ID NO: 3 or 4, to the extent that the fragments retain the substantial antigenicity of the parent polypeptide (specific antigenicity). Polypeptide derivatives can also be constructed by large internal deletions that remove a substantial part of the parent polypeptide, while retaining specific antigenicity. Generally, polypeptide derivatives should be about at least 12 amino acids in length to maintain antigenicity. Advantageously, they can be at least 20 amino acids, preferably at least 50 amino acids, more preferably at least 75 amino acids, and most preferably at least 100 amino acids in length.

Useful polypeptide derivatives, e.g., polypeptide fragments, can be designed using computer-assisted analysis of amino acid sequences in order to identify sites in protein antigens having potential as surface-exposed, antigenic regions (Ref 37).

Polypeptide fragments and polypeptides having large internal deletions can be used for revealing epitopes that are otherwise masked in the parent polypeptide and that may be of importance for inducing a protective T cell-dependent immune response. Deletions can also remove immunodominant regions of high variability among strains.

It is an accepted practice in the field of immunology to use fragments and variants of protein immunogens as vaccines, as all that is required to induce an immune response to a protein is a small (e.g., 8 to 10 amino acid) immunogenic region of the protein. This has been done for a number of vaccines against pathogens other than *Chlamydia*. For example, short

synthetic peptides corresponding to surface-exposed antigens of pathogens such as murine mammary tumor virus, peptide containing 11 amino acids; (Ref 38), Semliki Forest virus, peptide containing 16 amino acids (Ref 39), and canine parvovirus, 2 overlapping peptides, each containing 15 amino acids (Ref 40), have been shown to be effective vaccine antigens against their respective pathogens.

Polynucleotides encoding polypeptide fragments and polypeptides having large internal deletions can be constructed using standard methods (Ref 41), for example, by PCR, including inverse PCR, by restriction enzyme treatment of the cloned DNA molecules, or by the method of Kunkel *et al.* (Ref 42) biological material available at Stratagene.

A polypeptide derivative can also be produced as a fusion polypeptide that contains a polypeptide or a polypeptide derivative of the invention fused, *e.g.*, at the N- or C-terminal end, to any other polypeptide (hereinafter referred to as a peptide tail). Such a product can be easily obtained by translation of a genetic fusion, *i.e.*, a hybrid gene. Vectors for expressing fusion polypeptides are commercially available, such as the pMal-c2 or pMal-p2 systems of New England Biolabs, in which the peptide tail is a maltose binding protein, the glutathione-S-transferase system of Pharmacia, or the His-Tag system available from Novagen. These and other expression systems provide convenient means for further purification of polypeptides and derivatives of the invention.

Another particular example of fusion polypeptides included in invention includes a polypeptide or polypeptide derivative of the invention fused to a polypeptide having adjuvant activity, such as, *e.g.*, subunit B of either cholera toxin or *E. coli* heat-labile toxin. Several possibilities are can be used for achieving fusion. First, the polypeptide of the invention can be fused to the N-, or preferably, to the C-terminal end of the polypeptide having adjuvant activity. Second, a polypeptide fragment of the invention can be fused within the amino acid sequence of the polypeptide having adjuvant activity.

As stated above, the polynucleotides of the invention encode *Chlamydia* polypeptides in precursor or mature form. They can also encode hybrid precursors containing heterologous signal peptides, which can mature into polypeptides of the invention. By "heterologous signal peptide" is meant a signal peptide that is not found in the naturally-occurring precursor of a polypeptide of the invention.

A polynucleotide of the invention, having a homologous coding sequence, hybridizes, preferably under stringent conditions, to a polynucleotide having a sequence as shown in SEQ ID NOs:1 to 2. Hybridization procedures are, *e.g.*, described in Ausubel *et al.*, (Ref 41), Silhavy *et al.* (Ref 43); Davis *et al.* (ref 44). Important parameters that can be considered for optimizing hybridization conditions are reflected in a formula that allows calculation of a critical value, the melting temperature above which two complementary DNA strands separate from each other Ref 45). This formula is as follows: $T_m = 81.5 + 0.5 \times (\% \text{ G+C}) + 1.6 \log (\text{positive ion concentration}) - 0.6 \times (\% \text{ formamide})$. Under appropriate stringency conditions, hybridization temperature (T_h) is approximately 20 to 40°C, 20 to 25°C, or, preferably 30 to 40°C below the calculated T_m . Those skilled in the art will understand that optimal temperature and salt conditions can be readily determined empirically in preliminary experiments using conventional procedures.

For example, stringent conditions can be achieved, both for pre-hybridizing and hybridizing incubations, (i) within 4-16 hours at 42°C, in 6 x SSC containing 50% formamide or (ii) within 4-16 hours at 65°C in an aqueous 6 x SSC solution (1 M NaCl, 0.1 M sodium citrate (pH 7.0)).

For polynucleotides containing 30 to 600 nucleotides, the above formula is used and then is corrected by subtracting (600/polynucleotide size in base pairs). Stringency conditions are defined by a T_h that is 5 to 10°C below T_m .

Hybridization conditions with oligonucleotides shorter than 20-30 bases do not exactly follow the rules set forth above. In such cases, the formula for calculating the T_m is as follows: $T_m = 4 \times (\text{G+C}) + 2 (\text{A+T})$. For example, an 18 nucleotide fragment of 50% G+C would have an approximate T_m of 54°C.

A polynucleotide molecule of the invention, containing RNA, DNA, or modifications or combinations thereof, can have various applications. For example, a DNA molecule can be used (i) in a process for producing the encoded polypeptide in a recombinant host system, (ii) in the construction of vaccine vectors such as poxviruses, which are further used in methods and compositions for preventing and/or treating *Chlamydia* infection, (iii) as a vaccine agent (as well as an RNA molecule), in a naked form or formulated with a delivery vehicle and, (iv) in the construction of attenuated *Chlamydia* strains that can over-express a polynucleotide of the invention or express it in a non-toxic, mutated form.

According to a second aspect of the invention, there is therefore provided (i) an expression cassette containing a DNA molecule of the invention placed under the control of the elements required for expression, in particular under the control of an appropriate promoter; (ii) an expression vector containing an expression cassette of the invention; (iii) a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, as well as (iv) a process for producing a polypeptide or polypeptide derivative encoded by a polynucleotide of the invention, which involves culturing a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, under conditions that allow expression of the DNA molecule of the invention and, recovering the encoded polypeptide or polypeptide derivative from the cell culture.

A recombinant expression system can be selected from procaryotic and eucaryotic hosts. Eucaryotic hosts include yeast cells (*e.g.*, *Saccharomyces cerevisiae* or *Pichia pastoris*), mammalian cells (*e.g.*, COS1, NIH3T3, or JEG3 cells), arthropods cells (*e.g.*, *Spodoptera frugiperda* (SF9) cells), and plant cells. Preferably, a procaryotic host such as *E. coli* is used. Bacterial and eucaryotic cells are available from a number of different sources to those skilled in the art, *e.g.*, the American Type Culture Collection (ATCC; Rockville, Maryland).

The choice of the expression system depends on the features desired for the expressed polypeptide. For example, it may be useful to produce a polypeptide of the invention in a particular lipidated form or any other form.

The choice of the expression cassette will depend on the host system selected as well as the features desired for the expressed polypeptide. Typically, an expression cassette includes a promoter that is functional in the selected host system and can be constitutive or inducible; a ribosome binding site; a start codon (ATG) if necessary, a region encoding a signal peptide, *e.g.*, a lipidation signal peptide; a DNA molecule of the invention; a stop codon; and optionally a 3' terminal region (translation and/or transcription terminator). The signal peptide encoding region is adjacent to the polynucleotide of the invention and placed in proper reading frame. The signal peptide-encoding region can be homologous or heterologous to the DNA molecule encoding the mature polypeptide and can be specific to the secretion apparatus of the host used for expression. The open reading frame constituted by the DNA molecule of the invention, solely or together with the signal peptide, is placed under the control of the promoter so that transcription and translation occur in the host system. Promoters, signal peptide encoding regions are widely

known and available to those skilled in the art and includes, for example, the promoter of *Salmonella typhimurium* (and derivatives) that is inducible by arabinose (promoter araB) and is functional in Gram-negative bacteria such as *E. coli* (as described in U.S. Patent No. 5,028,530 and in Cagnon *et al.*, (Ref 46); the promoter of the gene of bacteriophage T7 encoding RNA polymerase, that is functional in a number of *E. coli* strains expressing T7 polymerase (described in U.S. Patent No. 4,952,496); OspA lipitation signal peptide ; and RlpB lipitation signal peptide (Ref 47).

The expression cassette is typically part of an expression vector, which is selected for its ability to replicate in the chosen expression system. Expression vectors (*e.g.*, plasmids or viral vectors) can be chosen from those described in Pouwels *et al.* (Cloning Vectors: A Laboratory Manual 1985, Supp. 1987). They can be purchased from various commercial sources.

Methods for transforming/transfecting host cells with expression vectors will depend on the host system selected as described in Ausubel *et al.*, (Ref 41).

Upon expression, a recombinant polypeptide of the invention (or a polypeptide derivative) is produced and remains in the intracellular compartment, is secreted/excreted in the extracellular medium or in the periplasmic space, or is embedded in the cellular membrane. The polypeptide can then be recovered in a substantially purified form from the cell extract or from the supernatant after centrifugation of the recombinant cell culture. Typically, the recombinant polypeptide can be purified by antibody-based affinity purification or by any other method that can be readily adapted by a person skilled in the art, such as by genetic fusion to a small affinity binding domain. Antibody-based affinity purification methods are also available for purifying a polypeptide of the invention extracted from a *Chlamydia* strain. Antibodies useful for purifying by immunoaffinity the polypeptides of the invention can be obtained as described below.

A polynucleotide of the invention can also be useful in the vaccine field, *e.g.*, for achieving DNA vaccination. There are two major possibilities, either using a viral or bacterial host as gene delivery vehicle (live vaccine vector) or administering the gene in a free form, *e.g.*, inserted into a plasmid. Therapeutic or prophylactic efficacy of a polynucleotide of the invention can be evaluated as described below.

Accordingly, in a third aspect of the invention, there is provided (i) a vaccine vector such as a poxvirus, containing a DNA molecule of the invention, placed under the control of

elements required for expression; (ii) a composition of matter containing a vaccine vector of the invention, together with a diluent or carrier; particularly, (iii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a vaccine vector of the invention; (iv) a method for inducing an immune response against *Chlamydia* in a mammal (e.g., a human; alternatively, the method can be used in veterinary applications for treating or preventing *Chlamydia* infection of animals, e.g., cats or birds), which involves administering to the mammal an immunogenically effective amount of a vaccine vector of the invention to elicit an immune response, e.g., a protective or therapeutic immune response to *Chlamydia*; and particularly, (v) a method for preventing and/or treating a *Chlamydia* (e.g., *C. trachomatis*, *C. psittaci*, *C. pneumonia*, *C. pecorum*) infection, which involves administering a prophylactic or therapeutic amount of a vaccine vector of the invention to an individual in need. Additionally, the third aspect of the invention encompasses the use of a vaccine vector of the invention in the preparation of a medicament for preventing and/or treating *Chlamydia* infection.

A vaccine vector of the invention can express one or several polypeptides or derivatives of the invention, as well as at least one additional *Chlamydia* antigen, fragment, homolog, mutant, or derivative thereof. In addition, it can express a cytokine, such as interleukin-2 (IL-2) or interleukin-12 (IL-12), that enhances the immune response (adjuvant effect). Thus, a vaccine vector can include an additional DNA sequence encoding, e.g., a chlamydial antigen, or a cytokine, placed under the control of elements required for expression in a mammalian cell.

Alternatively, a composition of the invention can include several vaccine vectors, each of them being capable of expressing a polypeptide or derivative of the invention. A composition can also contain a vaccine vector capable of expressing an additional *Chlamydia* antigen, or a subunit, fragment, homolog, mutant, or derivative thereof; or a cytokine such as IL-2 or IL-12.

In vaccination methods for treating or preventing infection in a mammal, a vaccine vector of the invention can be administered by any conventional route in use in the vaccine field, particularly, to a mucosal (e.g., ocular, intranasal, oral, gastric, pulmonary, intestinal, rectal, vaginal, or urinary tract) surface or via the parenteral (e.g., subcutaneous, intradermal, intramuscular, intravenous, or intraperitoneal) route. Preferred routes depend upon the choice of the vaccine vector. The administration can be achieved in a single dose or repeated at intervals.

The appropriate dosage depends on various parameters understood by skilled artisans such as the vaccine vector itself, the route of administration or the condition of the mammal to be vaccinated (weight, age and the like).

Live vaccine vectors available in the art include viral vectors such as adenoviruses and poxviruses as well as bacterial vectors, *e.g.*, *Shigella*, *Salmonella*, *Vibrio cholerae*, *Lactobacillus*, Bacille bilié de Calmette-Guérin (BCG), and *Streptococcus*.

An example of an adenovirus vector, as well as a method for constructing an adenovirus vector capable of expressing a DNA molecule of the invention, are described in U.S. Patent No. 4,920,209. Poxvirus vectors that can be used include, *e.g.*, vaccinia and canary pox virus, described in U.S. Patent No. 4,722,848 and U.S. Patent No. 5,364,773, respectively (also see, *e.g.*, Tartaglia *et al.*, Virology (1992) 188:217) for a description of a vaccinia virus vector; and Taylor *et al.*, Vaccine (1995) 13:539 for a reference of a canary pox). Poxvirus vectors capable of expressing a polynucleotide of the invention can be obtained by homologous recombination as described in Kieny *et al.*, Nature (1984) 312:163 so that the polynucleotide of the invention is inserted in the viral genome under appropriate conditions for expression in mammalian cells. Generally, the dose of vaccine viral vector, for therapeutic or prophylactic use, can be of from about 1×10^4 to about 1×10^{11} , advantageously from about 1×10^7 to about 1×10^{10} , preferably of from about 1×10^7 to about 1×10^9 plaque-forming units per kilogram. Preferably, ~~viral vectors are administered parenterally, for example, in 3 doses, 4 weeks apart. Those skilled~~ in the art recognize that it is preferable to avoid adding a chemical adjuvant to a composition containing a viral vector of the invention and thereby minimizing the immune response to the viral vector itself.

Non-toxicogenic *Vibrio cholerae* mutant strains that are useful as a live oral vaccine are described in Mekalanos *et al.*, Nature (1983) 306:551 and U.S. Patent No. 4,882,278 (strain in which a substantial amount of the coding sequence of each of the two *ctxA* alleles has been deleted so that no functional *cholerae* toxin is produced); WO 92/11354 (strain in which the *irgA* locus is inactivated by mutation; this mutation can be combined in a single strain with *ctxA* mutations); and WO 94/1533 (deletion mutant lacking functional *ctxA* and *attRSI* DNA sequences). These strains can be genetically engineered to express heterologous antigens, as described in WO 94/19482. An effective vaccine dose of a *Vibrio cholerae* strain capable of expressing a polypeptide or polypeptide derivative encoded by a DNA molecule of the invention

can contain, e.g., about 1×10^5 to about 1×10^9 , preferably about 1×10^6 to about 1×10^8 viable bacteria in an appropriate volume for the selected route of administration. Preferred routes of administration include all mucosal routes; most preferably, these vectors are administered intranasally or orally.

5 ~~Attenuated *Salmonella typhimurium* strains, genetically engineered for recombinant expression of heterologous antigens or not, and their use as oral vaccines are described in Nakayama et al., (Bio/Technology (1988) 6:693) and WO 92/11361. Preferred routes of administration include all mucosal routes; most preferably, these vectors are administered intranasally or orally.~~

10 Others bacterial strains useful as vaccine vectors are described in High et al., EMBO (1992) 11:1991 and Sizemore et al., Science (1995) 270:299 (*Shigella flexneri*); Medaglini et al., Proc. Natl. Acad. Sci. USA (1995) 92:6868 (*Streptococcus gordonii*); and Flynn J.L., Cell. Mol. Biol. (1994) 40 (suppl. I):31, WO 88/6626, WO 90/0594, WO 91/13157, WO 92/1796, and WO 92/21376 (Bacille Calmette Guerin).

15 ~~In bacterial vectors, polynucleotide of the invention can be inserted into the bacterial genome or can remain in a free state, carried on a plasmid.~~

~~An adjuvant can also be added to a composition containing a vaccine bacterial vector. A number of adjuvants are known to those skilled in the art. Preferred adjuvants can be selected from the list provided below.~~

20 ~~According to a fourth aspect of the invention, there is also provided (i) a composition of matter containing a polynucleotide of the invention, together with a diluent or carrier; (ii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a polynucleotide of the invention; (iii) a method for inducing an immune response against *Chlamydia*, in a mammal, by administering to the mammal, an immunogenically effective~~
25 ~~amount of a polynucleotide of the invention to elicit an immune response, e.g., a protective immune response to *Chlamydia*; and particularly, (iv) a method for preventing and/or treating a *Chlamydia* (e.g., *C. trachomatis*, *C. psittaci*, *C. pneumoniae*, or *C. pecorum*) infection, by administering a prophylactic or therapeutic amount of a polynucleotide of the invention to an individual in need. Additionally, the fourth aspect of the invention encompasses the use of a~~
30 ~~polynucleotide of the invention in the preparation of a medicament for preventing and/or treating *Chlamydia* infection. The fourth aspect of the invention preferably includes the use of a DNA~~

molecule placed under conditions for expression in a mammalian cell, *e.g.*, in a plasmid that is unable to replicate in mammalian cells and to substantially integrate in a mammalian genome.

Polynucleotides (DNA or RNA) of the invention can also be administered as such to a mammal for vaccine, *e.g.*, therapeutic or prophylactic, purpose. When a DNA molecule of the invention is used, it can be in the form of a plasmid that is unable to replicate in a mammalian cell and unable to integrate in the mammalian genome. Typically, a DNA molecule is placed under the control of a promoter suitable for expression in a mammalian cell. The promoter can function ubiquitously or tissue-specifically. Examples of non-tissue specific promoters include the early Cytomegalovirus (CMV) promoter (described in U.S. Patent No. 4,168,062) and the Rous Sarcoma Virus promoter (described in Norton & Coffin, *Molec. Cell Biol.* (1985) 5:281). The desmin promoter (Li *et al.*, *Gene* (1989) 78:243, Li & Paulin, *J. Biol. Chem.* (1991) 266:6562 and Li & Paulin, *J. Biol. Chem.* (1993) 268:10403) is tissue-specific and drives expression in muscle cells. More generally, useful vectors are described, *i.a.*, WO 94/21797 and Hartikka *et al.*, *Human Gene Therapy* (1996) 7:1205.

For DNA/RNA vaccination, the polynucleotide of the invention can encode a precursor or a mature form. When it encodes a precursor form, the precursor form can be homologous or heterologous. In the latter case, a eucaryotic leader sequence can be used, such as the leader sequence of the tissue-type plasminogen factor (tPA).

A composition of the invention can contain one or several polynucleotides of the invention. It can also contain at least one additional polynucleotide encoding another *Chlamydia* antigen such as urease subunit A, B, or both; or a fragment, derivative, mutant, or analog thereof. A polynucleotide encoding a cytokine, such as interleukin-2 (IL-2) or interleukin-12 (IL-12), can also be added to the composition so that the immune response is enhanced. These additional polynucleotides are placed under appropriate control for expression. Advantageously, DNA molecules of the invention and/or additional DNA molecules to be included in the same composition, can be carried in the same plasmid.

Standard techniques of molecular biology for preparing and purifying polynucleotides can be used in the preparation of polynucleotide therapeutics of the invention. For use as a vaccine, a polynucleotide of the invention can be formulated according to various methods.

First, a polynucleotide can be used in a naked form, free of any delivery vehicles, such as anionic liposomes, cationic lipids, microparticles, *e.g.*, gold microparticles, precipitating

agents, *e.g.*, calcium phosphate, or any other transfection-facilitating agent. In this case, the polynucleotide can be simply diluted in a physiologically acceptable solution, such as sterile saline or sterile buffered saline, with or without a carrier. When present, the carrier preferably is isotonic, hypotonic, or weakly hypertonic, and has a relatively low ionic strength, such as provided by a sucrose solution, *e.g.*, a solution containing 20% sucrose.

Alternatively, a polynucleotide can be associated with agents that assist in cellular uptake. It can be, *i.a.*, (i) complemented with a chemical agent that modifies the cellular permeability, such as bupivacaine (see, *e.g.*, WO 94/16737), (ii) encapsulated into liposomes, or (iii) associated with cationic lipids or silica, gold, or tungsten microparticles.

Anionic and neutral liposomes are well-known in the art (see, *e.g.*, Liposomes: A Practical Approach, RPC New Ed, IRL press (1990), for a detailed description of methods for making liposomes) and are useful for delivering a large range of products, including polynucleotides.

Cationic lipids are also known in the art and are commonly used for gene delivery. Such lipids include Lipofectin™ also known as DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride), DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane), DDAB (dimethyldioctadecylammonium bromide), DOGS (dioctadecylamidoglycyl spermine) and cholesterol derivatives such as DC-Chol (3- β -(N,N'-dimethylaminomethane)-carbamoyl) cholesterol). A description of these cationic lipids can be found in EP 187,702, WO 90/11092, U.S. Patent No. 5,283,185, WO 91/15501, WO 95/26356, and U.S. Patent No. 5,527,928. Cationic lipids for gene delivery are preferably used in association with a neutral lipid such as DOPE (dioleyl phosphatidylethanolamine), as, for example, described in WO 90/11092.

Other transfection-facilitating compounds can be added to a formulation containing cationic liposomes. A number of them are described in, *e.g.*, WO 93/18759, WO 93/19768, WO 94/25608, and WO 95/2397. They include, *i.a.*, spermine derivatives useful for facilitating the transport of DNA through the nuclear membrane (see, for example, WO 93/18759) and membrane-permeabilizing compounds such as GALA, Gramicidine S, and cationic bile salts (see, for example, WO 93/19768).

Gold or tungsten microparticles can also be used for gene delivery, as described in WO 91/359, WO 93/17706, and Tang *et al.* (Nature (1992) 356:152). In this case, the

microparticle-coated polynucleotides can be injected *via* intradermal or intraepidermal routes using a needleless injection device ("gene gun"), such as those described in U.S. Patent No. 4,945,050, U.S. Patent No. 5,015,580, and WO 94/24263.

The amount of DNA to be used in a vaccine recipient depends, *e.g.*, on the strength of the promoter used in the DNA construct, the immunogenicity of the expressed gene product, the condition of the mammal intended for administration (*e.g.*, the weight, age, and general health of the mammal), the mode of administration, and the type of formulation. In general, a therapeutically or prophylactically effective dose from about 1 μ g to about 1 mg, preferably, from about 10 μ g to about 800 μ g and, more preferably, from about 25 μ g to about 250 μ g, can be administered to human adults. The administration can be achieved in a single dose or repeated at intervals.

The route of administration can be any conventional route used in the vaccine field. As general guidance, a polynucleotide of the invention can be administered *via* a mucosal surface, *e.g.*, an ocular, intranasal, pulmonary, oral, intestinal, rectal, vaginal, and urinary tract surface; or *via* a parenteral route, *e.g.*, by an intravenous, subcutaneous, intraperitoneal, intradermal, intraepidermal, or intramuscular route. The choice of the administration route will depend on, *e.g.*, the formulation that is selected. A polynucleotide formulated in association with bupivacaine is advantageously administered into muscles. When a neutral or anionic liposome or a cationic lipid, such as DOTMA or DC-Chol, is used, the formulation can be advantageously injected *via* intravenous, intranasal (aerosolization), intramuscular, intradermal, and subcutaneous routes. A polynucleotide in a naked form can advantageously be administered *via* the intramuscular, intradermal, or sub-cutaneous routes.

Although not absolutely required, such a composition can also contain an adjuvant. If so, a systemic adjuvant that does not require concomitant administration in order to exhibit an adjuvant effect is preferable such as, *e.g.*, QS21, which is described in U.S. Patent No. 5,057,546.

The sequence information provided in the present application enables the design of specific nucleotide probes and primers that can be useful in diagnosis. Accordingly, in a fifth aspect of the invention, there is provided a nucleotide probe or primer having a sequence found in or derived by degeneracy of the genetic code from a sequence shown in SEQ ID NO:1 to 2.

The term "probe" as used in the present application refers to DNA (preferably single stranded) or RNA molecules (or modifications or combinations thereof) that hybridize under the

stringent conditions, as defined above, to nucleic acid molecules having sequences homologous to those shown in SEQ ID NOs:1 and 2, or to a complementary or anti-sense sequence. Generally, probes are significantly shorter than full-length sequences shown in SEQ ID NOs:1 and 2; for example, they can contain from about 5 to about 100, preferably from about 10 to about 80 nucleotides. In particular, probes have sequences that are at least 75%, preferably at least 85%, more preferably 95% homologous to a portion of a sequence as shown in SEQ ID NOs:1 and 2 or that are complementary to such sequences. Probes can contain modified bases such as inosine, methyl-5-deoxycytidine, deoxyuridine, dimethylamino-5-deoxyuridine, or diamino-2, 6-purine. Sugar or phosphate residues can also be modified or substituted. For example, a deoxyribose residue can be replaced by a polyamide (Nielsen *et al.*, Science (1991) 254:1497) and phosphate residues can be replaced by ester groups such as diphosphate, alkyl, arylphosphonate and phosphorothioate esters. In addition, the 2'-hydroxyl group on ribonucleotides can be modified by including, *e.g.*, alkyl groups.

Probes of the invention can be used in diagnostic tests, as capture or detection probes. Such capture probes can be conventionally immobilized on a solid support, directly or indirectly, by covalent means or by passive adsorption. A detection probe can be labelled by a detection marker selected from radioactive isotopes, enzymes such as peroxidase, alkaline phosphatase, and enzymes able to hydrolyze a chromogenic, fluorogenic, or luminescent substrate, compounds that are chromogenic, fluorogenic, or luminescent, nucleotide base analogs, and biotin.

Probes of the invention can be used in any conventional hybridization technique, such as dot blot (Maniatis *et al.*, Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), Southern blot (Southern, J. Mol. Biol. (1975) 98:503), northern blot (identical to Southern blot to the exception that RNA is used as a target), or the sandwich technique (Dunn *et al.*, Cell (1977) 12:23). The latter technique involves the use of a specific capture probe and/or a specific detection probe with nucleotide sequences that at least partially differ from each other.

A primer is usually a probe of about 10 to about 40 nucleotides that is used to initiate enzymatic polymerization of DNA in an amplification process (*e.g.*, PCR), in an elongation process, or in a reverse transcription method. In a diagnostic method involving PCR, primers can be labelled.

Thus, the invention also encompasses (i) a reagent containing a probe of the invention for detecting and/or identifying the presence of *Chlamydia* in a biological material; (ii) a method for detecting and/or identifying the presence of *Chlamydia* in a biological material, in which (a) a sample is recovered or derived from the biological material, (b) DNA or RNA is extracted from the material and denatured, and (c) exposed to a probe of the invention, for example, a capture, detection probe or both, under stringent hybridization conditions, such that hybridization is detected; and (iii) a method for detecting and/or identifying the presence of *Chlamydia* in a biological material, in which (a) a sample is recovered or derived from the biological material, (b) DNA is extracted therefrom, (c) the extracted DNA is primed with at least one, and preferably two, primers of the invention and amplified by polymerase chain reaction, and (d) the amplified DNA fragment is produced.

As previously mentioned, polypeptides that can be produced upon expression of the newly identified open reading frames are useful vaccine agents.

Therefore, a sixth aspect of the invention features a substantially purified polypeptide or polypeptide derivative having an amino acid sequence encoded by a polynucleotide of the invention.

A "substantially purified polypeptide" is defined as a polypeptide that is separated from the environment in which it naturally occurs and/or that is free of the majority of the polypeptides that are present in the environment in which it was synthesized. For example, a substantially purified polypeptide is free from cytoplasmic polypeptides. Those skilled in the art will understand that the polypeptides of the invention can be purified from a natural source, *i.e.*, a *Chlamydia* strain, or can be produced by recombinant means.

Homologous polypeptides or polypeptide derivatives encoded by polynucleotides of the invention can be screened for specific antigenicity by testing cross-reactivity with an antiserum raised against the polypeptide of reference having an amino acid sequence as shown in SEQ ID NOs:3 to 4. Briefly, a monospecific hyperimmune antiserum can be raised against a purified reference polypeptide as such or as a fusion polypeptide, for example, an expression product of MBP, GST, or His-tag systems or a synthetic peptide predicted to be antigenic. The homologous polypeptide or derivative screened for specific antigenicity can be produced as such or as a fusion polypeptide. In this latter case and if the antiserum is also raised against a fusion polypeptide, two different fusion systems are employed. Specific antigenicity can be determined

according to a number of methods, including Western blot (Towbin *et al.*, Proc. Natl. Acad. Sci. USA (1979) 76:4350), dot blot, and ELISA, as described below.

In a Western blot assay, the product to be screened, either as a purified preparation or a total *E. coli* extract, is submitted to SDS-Page electrophoresis as described by Laemmli (Nature (1970) 227:680). After transfer to a nitrocellulose membrane, the material is further incubated with the monospecific hyperimmune antiserum diluted in the range of dilutions from about 1:5 to about 1:5000, preferably from about 1:100 to about 1:500. Specific antigenicity is shown once a band corresponding to the product exhibits reactivity at any of the dilutions in the above range.

In an ELISA assay, the product to be screened is preferably used as the coating antigen. A purified preparation is preferred, although a whole cell extract can also be used. Briefly, about 100 μ l of a preparation at about 10 μ g protein/ml are distributed into wells of a 96-well polycarbonate ELISA plate. The plate is incubated for 2 hours at 37°C then overnight at 4°C. The plate is washed with phosphate buffer saline (PBS) containing 0.05% Tween 20 (PBS/Tween buffer). The wells are saturated with 250 μ l PBS containing 1% bovine serum albumin (BSA) to prevent non-specific antibody binding. After 1 hour incubation at 37°C, the plate is washed with PBS/Tween buffer. The antiserum is serially diluted in PBS/Tween buffer containing 0.5% BSA. 100 μ l of dilutions are added per well. The plate is incubated for 90 minutes at 37°C, washed and evaluated according to standard procedures. For example, a goat anti-rabbit peroxidase conjugate is added to the wells when specific antibodies were raised in rabbits. Incubation is carried out for 90 minutes at 37°C and the plate is washed. The reaction is developed with the appropriate substrate and the reaction is measured by colorimetry (absorbance measured spectrophotometrically). Under the above experimental conditions, a positive reaction is shown by O.D. values greater than a non immune control serum.

In a dot blot assay, a purified product is preferred, although a whole cell extract can also be used. Briefly, a solution of the product at about 100 μ g/ml is serially two-fold diluted in 50 mM Tris-HCl (pH 7.5). 100 μ l of each dilution are applied to a nitrocellulose membrane 0.45 μ m set in a 96-well dot blot apparatus (Biorad). The buffer is removed by applying vacuum to the system. Wells are washed by addition of 50 mM Tris-HCl (pH 7.5) and the membrane is air-dried. The membrane is saturated in blocking buffer (50 mM Tris-HCl (pH 7.5) 0.15 M NaCl, 10 g/L skim milk) and incubated with an antiserum dilution from about 1:50 to about 1:5000, preferably about 1:500. The reaction is revealed according to standard procedures. For example,

a goat anti-rabbit peroxidase conjugate is added to the wells when rabbit antibodies are used. Incubation is carried out 90 minutes at 37°C and the blot is washed. The reaction is developed with the appropriate substrate and stopped. The reaction is measured visually by the appearance of a colored spot, *e.g.*, by colorimetry. Under the above experimental conditions, a positive reaction is shown once a colored spot is associated with a dilution of at least about 1:5, preferably of at least about 1:500.

Therapeutic or prophylactic efficacy of a polypeptide or derivative of the invention can be evaluated as described below.

According to a seventh aspect of the invention, there is provided (i) a composition of matter containing a polypeptide of the invention together with a diluent or carrier; in particular, (ii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a polypeptide of the invention; (iii) a method for inducing an immune response against *Chlamydia* in a mammal, by administering to the mammal an immunogenically effective amount of a polypeptide of the invention to elicit an immune response, *e.g.*, a protective immune response to *Chlamydia*; and particularly, (iv) a method for preventing and/or treating a *Chlamydia* (*e.g.*, *C. trachomatis*, *C. psittaci*, *C. pneumoniae*, or *C. pecorum*) infection, by administering a prophylactic or therapeutic amount of a polypeptide of the invention to an individual in need. Additionally, the seventh aspect of the invention encompasses the use of a polypeptide of the invention in the preparation of a medicament for preventing and/or treating *Chlamydia* infection.

The immunogenic compositions of the invention can be administered by any conventional route in use in the vaccine field, in particular to a mucosal (*e.g.*, ocular, intranasal, pulmonary, oral, gastric, intestinal, rectal, vaginal, or urinary tract) surface or *via* the parenteral (*e.g.*, subcutaneous, intradermal, intramuscular, intravenous, or intraperitoneal) route. The choice of the administration route depends upon a number of parameters, such as the adjuvant associated with the polypeptide. For example, if a mucosal adjuvant is used, the intranasal or oral route will be preferred and if a lipid formulation or an aluminum compound is used, the parenteral route will be preferred. In the latter case, the sub-cutaneous or intramuscular route is most preferred. The choice can also depend upon the nature of the vaccine agent. For example, a polypeptide of the invention fused to CTB or LTB will be best administered to a mucosal surface.

A composition of the invention can contain one or several polypeptides or derivatives of the invention. It can also contain at least one additional *Chlamydia* antigen, or a subunit, fragment, homolog, mutant, or derivative thereof.

For use in a composition of the invention, a polypeptide or derivative thereof can be formulated into or with liposomes, preferably neutral or anionic liposomes, microspheres, ISCOMS, or virus-like-particles (VLPs) to facilitate delivery and/or enhance the immune response. These compounds are readily available to one skilled in the art; for example, see Liposomes: A Practical Approach (*supra*).

Adjuvants other than liposomes and the like can also be used and are known in the art. A appropriate selection can conventionally be made by those skilled in the art, for example, from the list provided below.

Administration can be achieved in a single dose or repeated as necessary at intervals as can be determined by one skilled in the art. For example, a priming dose can be followed by three booster doses at weekly or monthly intervals. An appropriate dose depends on various parameters including the recipient (*e.g.*, adult or infant), the particular vaccine antigen, the route and frequency of administration, the presence/absence or type of adjuvant, and the desired effect (*e.g.*, protection and/or treatment), as can be determined by one skilled in the art. In general, a vaccine antigen of the invention can be administered by a mucosal route in an amount from about 10 μ g to about 500 mg, preferably from about 1 mg to about 200 mg. For the parenteral route of administration, the dose usually should not exceed about 1 mg, preferably about 100 μ g.

When used as vaccine agents, polynucleotides and polypeptides of the invention can be used sequentially as part of a multistep immunization process. For example, a mammal can be initially primed with a vaccine vector of the invention such as a pox virus, *e.g.*, via the parenteral route, and then boosted twice with the polypeptide encoded by the vaccine vector, *e.g.*, via the mucosal route. In another example, liposomes associated with a polypeptide or derivative of the invention can also be used for priming, with boosting being carried out mucosally using a soluble polypeptide or derivative of the invention in combination with a mucosal adjuvant (*e.g.*, LT).

A polypeptide derivative of the invention is also useful as a diagnostic reagent for detecting the presence of anti-*Chlamydia* antibodies, *e.g.*, in a blood sample. Such polypeptides are about 5 to about 80, preferably about 10 to about 50 amino acids in length and can be labeled

or unlabeled, depending upon the diagnostic method. Diagnostic methods involving such a reagent are described below.

Upon expression of a DNA molecule of the invention, a polypeptide or polypeptide derivative is produced and can be purified using known laboratory techniques. For example, the polypeptide or polypeptide derivative can be produced as a fusion protein containing a fused tail that facilitates purification. The fusion product can be used to immunize a small mammal, *e.g.*, a mouse or a rabbit, in order to raise antibodies against the polypeptide or polypeptide derivative (monospecific antibodies). The eighth aspect of the invention thus provides a monospecific antibody that binds to a polypeptide or polypeptide derivative of the invention.

By "monospecific antibody" is meant an antibody that is capable of reacting with a unique naturally-occurring *Chlamydia* polypeptide. An antibody of the invention can be polyclonal or monoclonal. Monospecific antibodies can be recombinant, *e.g.*, chimeric (*e.g.*, constituted by a variable region of murine origin associated with a human constant region), humanized (a human immunoglobulin constant backbone together with hypervariable region of animal, *e.g.*, murine, origin), and/or single chain. Both polyclonal and monospecific antibodies can also be in the form of immunoglobulin-fragments, *e.g.*, F(ab)'2 or-Fab fragments. The antibodies of the invention can be of any isotype, *e.g.*, IgG or IgA, and polyclonal antibodies can be of a single isotype or can contain a mixture of isotypes.

The antibodies of the invention, which are raised to a polypeptide or polypeptide derivative of the invention, can be produced and identified using standard immunological assays, *e.g.*, Western blot analysis, dot blot assay, or ELISA (see, *e.g.*, Coligan *et al.*, Current Protocols in Immunology (1994) John Wiley & Sons, Inc., New York, NY). The antibodies can be used in diagnostic methods to detect the presence of a *Chlamydia* antigen in a sample, such as a biological sample. The antibodies can also be used in affinity chromatography methods for purifying a polypeptide or polypeptide derivative of the invention. As is discussed further below, such antibodies can be used in prophylactic and therapeutic passive immunization methods.

Accordingly, a ninth aspect of the invention provides (i) a reagent for detecting the presence of *Chlamydia* in a biological sample that contains an antibody, polypeptide, or polypeptide derivative of the invention; and (ii) a diagnostic method for detecting the presence of *Chlamydia* in a biological sample, by contacting the biological sample with an antibody, a

polypeptide, or a polypeptide derivative of the invention, such that an immune complex is formed, and by detecting such complex to indicate the presence of *Chlamydia* in the sample or the organism from which the sample is derived.

Those skilled in the art will understand that the immune complex is formed between a component of the sample and the antibody, polypeptide, or polypeptide derivative, whichever is used, and that any unbound material can be removed prior to detecting the complex. As can be easily understood, a polypeptide reagent is useful for detecting the presence of anti-*Chlamydia* antibodies in a sample, e.g., a blood sample, while an antibody of the invention can be used for screening a sample, such as a gastric extract or biopsy, for the presence of *Chlamydia* polypeptides.

For use in diagnostic applications, the reagent (i.e., the antibody, polypeptide, or polypeptide derivative of the invention) can be in a free state or immobilized on a solid support, such as a tube, a bead, or any other conventional support used in the field. Immobilization can be achieved using direct or indirect means. Direct means include passive adsorption (non-covalent binding) or covalent binding between the support and the reagent. By "indirect means" is meant that an anti-reagent compound that interacts with a reagent is first attached to the solid support. For example, if a polypeptide reagent is used, an antibody that binds to it can serve as an anti-reagent, provided that it binds to an epitope that is not involved in the recognition of antibodies in biological samples. Indirect means can also employ a ligand-receptor system; for example, a molecule such as a vitamin can be grafted onto the polypeptide reagent and the corresponding receptor can be immobilized on the solid phase. This is illustrated by the biotin-streptavidin system. Alternatively, indirect means can be used, e.g., by adding to the reagent a peptide tail, chemically or by genetic engineering, and immobilizing the grafted or fused product by passive adsorption or covalent linkage of the peptide tail.

According to a tenth aspect of the invention, there is provided a process for purifying, from a biological sample, a polypeptide or polypeptide derivative of the invention, which involves carrying out antibody-based affinity chromatography with the biological sample, wherein the antibody is a monospecific antibody of the invention.

For use in a purification process of the invention, the antibody can be polyclonal or monospecific, and preferably is of the IgG type. Purified IgGs can be prepared from an antiserum using standard methods (see, e.g., Coligan *et al.*, *supra*). Conventional

chromatography supports, as well as standard methods for grafting antibodies, are disclosed in, *e.g.*, Antibodies: A Laboratory Manual, D. Lane, E. Harlow, Eds. (1988).

Briefly, a biological sample, such as an *C. pneumoniae* extract, preferably in a buffer solution, is applied to a chromatography material, preferably equilibrated with the buffer used to dilute the biological sample so that the polypeptide or polypeptide derivative of the invention (*i.e.*, the antigen) is allowed to adsorb onto the material. The chromatography material, such as a gel or a resin coupled to an antibody of the invention, can be in batch form or in a column. The unbound components are washed off and the antigen is then eluted with an appropriate elution buffer, such as a glycine buffer or a buffer containing a chaotropic agent, *e.g.*, guanidine HCl, or high salt concentration (*e.g.*, 3 M MgCl₂). Eluted fractions are recovered and the presence of the antigen is detected, *e.g.*, by measuring the absorbance at 280 nm.

An antibody of the invention can be screened for therapeutic efficacy as described as follows. According to an eleventh aspect of the invention, there is provided (i) a composition of matter containing a monospecific antibody of the invention, together with a diluent or carrier; (ii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a monospecific antibody of the invention, and (iii) a method for treating or preventing a *Chlamydia* (*e.g.*, *C. trachomatis*, *C. psittaci*, *C. pneumoniae* or *C. pecorum*) infection, by administering a therapeutic or prophylactic amount of a monospecific antibody of the invention to an individual in need. Additionally, the eleventh aspect of the invention encompasses the use of a monospecific antibody of the invention in the preparation of a medicament for treating or preventing *Chlamydia* infection.

To this end, the monospecific antibody can be polyclonal or monoclonal, preferably of the IgA isotype (predominantly). In passive immunization, the antibody can be administered to a mucosal surface of a mammal, *e.g.*, the gastric mucosa, *e.g.*, orally or intragastrically, advantageously, in the presence of a bicarbonate buffer. Alternatively, systemic administration, not requiring a bicarbonate buffer, can be carried out. A monospecific antibody of the invention can be administered as a single active component or as a mixture with at least one monospecific antibody specific for a different *Chlamydia* polypeptide. The amount of antibody and the particular regimen used can be readily determined by one skilled in the art. For example, daily administration of about 100 to 1,000 mg of antibodies over one week, or three doses per day of

about 100 to 1,000 mg of antibodies over two or three days, can be an effective regimens for most purposes.

Therapeutic or prophylactic efficacy can be evaluated using standard methods in the art, *e.g.*, by measuring induction of a mucosal immune response or induction of protective and/or therapeutic immunity, using, *e.g.*, the *C. pneumoniae* mouse model. Those skilled in the art will recognize that the *C. pneumoniae* strain of the model can be replaced with another *Chlamydia* strain. For example, the efficacy of DNA molecules and polypeptides from *C. pneumoniae* is preferably evaluated in a mouse model using an *C. pneumoniae* strain. Protection can be determined by comparing the degree of *Chlamydia* infection to that of a control group. Protection is shown when infection is reduced by comparison to the control group. Such an evaluation can be made for polynucleotides, vaccine vectors, polypeptides and derivatives thereof, as well as antibodies of the invention.

Adjuvants useful in any of the vaccine compositions described above are as follows.

Adjuvants for parenteral administration include aluminum compounds, such as aluminum hydroxide, aluminum phosphate, and aluminum hydroxy phosphate. The antigen can be precipitated with, or adsorbed onto, the aluminum compound according to standard protocols. Other adjuvants, such as RIBI (ImmunoChem, Hamilton, MT), can be used in parenteral administration.

Adjuvants for mucosal administration include bacterial toxins, *e.g.*, the cholera toxin (CT), the *E. coli* heat-labile toxin (LT), the *Clostridium difficile* toxin A and the pertussis toxin (PT), or combinations, subunits, toxoids, or mutants thereof. For example, a purified preparation of native cholera toxin subunit B (CTB) can be of use. Fragments, homologs, derivatives, and fusions to any of these toxins are also suitable, provided that they retain adjuvant activity. Preferably, a mutant having reduced toxicity is used. Suitable mutants are described, *e.g.*, in WO 95/17211 (Arg-7-Lys CT mutant), WO 96/6627 (Arg-192-Gly LT mutant), and WO 95/34323 (Arg-9-Lys and Glu-129-Gly PT mutant). Additional LT mutants that can be used in the methods and compositions of the invention include, *e.g.*, Ser-63-Lys, Ala-69-Gly, Glu-110-Asp, and Glu-112-Asp mutants. Other adjuvants, such as a bacterial monophosphoryl lipid A (MPLA) of, *e.g.*, *E. coli*, *Salmonella minnesota*, *Salmonella typhimurium*, or *Shigella flexneri*; saponins, or polylactide-glycolide (PLGA) microspheres, can also be used in mucosal administration.

Adjuvants useful for both mucosal and parenteral administrations include polyphosphazene (WO 95/2415), DC-chol (3 b-(N-(N',N'-dimethyl aminomethane)-carbamoyl) cholesterol; U.S. Patent No. 5,283,185 and WO 96/14831) and QS-21 (WO 88/9336).

Any pharmaceutical composition of the invention, containing a polynucleotide, a polypeptide, a polypeptide derivative, or an antibody of the invention, can be manufactured in a conventional manner. In particular, it can be formulated with a pharmaceutically acceptable diluent or carrier, e.g., water or a saline solution such as phosphate buffer saline. In general, a diluent or carrier can be selected on the basis of the mode and route of administration, and standard pharmaceutical practice. Suitable pharmaceutical carriers or diluents, as well as pharmaceutical necessities for their use in pharmaceutical formulations, are described in *Remington's Pharmaceutical Sciences*, a standard reference text in this field and in the USP/NF.

The invention also includes methods in which *Chlamydia* infection, are treated by oral administration of a *Chlamydia* polypeptide of the invention and a mucosal adjuvant, in combination with an antibiotic, an antacid, sucralfate, or a combination thereof. Examples of such compounds that can be administered with the vaccine antigen and the adjuvant are antibiotics, including, e.g., macrolides, tetracyclines, and derivatives thereof (specific examples of antibiotics that can be used include azithromycin or doxycyclin or immunomodulators such as cytokines or steroids. In addition, compounds containing more than one of the above-listed components coupled together, can be used. The invention also includes compositions for carrying out these methods, i.e., compositions containing a *Chlamydia* antigen (or antigens) of the invention, an adjuvant, and one or more of the above-listed compounds, in a pharmaceutically acceptable carrier or diluent.

Amounts of the above-listed compounds used in the methods and compositions of the invention can readily be determined by one skilled in the art. In addition, one skilled in the art can readily design treatment/immunization schedules. For example, the non-vaccine components can be administered on days 1-14, and the vaccine antigen + adjuvant can be administered on days 7, 14, 21, and 28.

REFERENCES

1. Grayston et al. (1995) Journal of Infectious Diseases 168:1231
2. Campos et al. (1995) Investigation of Ophthalmology and Visual Science 36:1477
3. Grayston et al (1990) Journal of Infectious Diseases 161:618
- 5 4. Marrie (1993) Clinical Infectious Diseases 18:501
5. Wang et al (1986) Chlamydial infections, Cambridge University Press, Cambridge, p. 329
6. Saikku et al. (1988) Lancet; ii:983
7. Thom et al. (1992) JAMA 268:68
8. Linnanmaki et al. (1993), Circulation 87:1030
- 10 9. Saikku et al. (1992) Annals Internal Medicine 116:273
10. Melnick et al (1993) American Journal of Medicine 95:499
- 11 Shor et al. (1992) South African. Medical Journal 82:158
12. Kuo et al. (1993) Journal of Infectious Diseases 167:841
13. Kuo et al. (1993) Arteriosclerosis and Thrombosis 13:1500
- 15 14. Campbell et al (1995) Journal of Infectious Diseases 172:585
15. Chiu et al. Circulation, 1997 (In Press).
16. Ramirez et al (1996) Annals of Internal Medicine 125:979
17. Jackson et al. Abst. K121, p272, 36th ICAAC, 15-18 Sept, 1996, New Orleans.
18. Fong et al (1997) Journal of Clinical Microbiology 35:48
- 20 19. Hahn DL, et al. Evidence for Chlamydia pneumoniae infection in steroid-dependent asthma.
Ann Allergy Asthma Immunol. 1998 Jan; 80(1): 45-49.
20. Hahn DL, et al. Association of Chlamydia pneumoniae IgA antibodies with recently symptomatic asthma. Epidemiol Infect. 1996 Dec; 117(3): 513-517.
21. Bjornsson E, et al. Serology of chlamydia in relation to asthma and bronchial hyperresponsiveness. Scand J Infect Dis. 1996; 28(1): 63-69.
- 25 22. Hahn DL. Treatment of Chlamydia pneumoniae infection in adult asthma: a before-after trial. J Fam Pract. 1995 Oct; 41(4): 345-351.
23. Allegra L, et al. Acute exacerbations of asthma in adults: role of Chlamydia pneumoniae infection. Eur Respir J. 1994 Dec; 7(12): 2165-2168.
- 30 24. Hahn DL, et al. Association of Chlamydia pneumoniae (strain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. JAMA. 1991 Jul 10; 266(2): 225-230.

25. Pal *et al.* (1996) *Infection and Immunity* 64:5341
26. Jones *et al.* (1995) *Vaccine* 13:715
27. Igietsemes *et al.* (1993) *Immunology* 5:317
28. Igietseme *et al.* (1993) *Regional Immunology* 5:317
- 5 29. Magee *et al.* (1993) *Regional Immunology* 5: 305
30. Landers *et al.* (1991) *Infection & Immunity* 59:3774
31. Magee *et al.* (1995) *Infection & Immunity* 63:516
32. Cotter *et al.* (1995) *Infection and Immunity* 63:4704
33. Campbell *et al.* (1990) *Infection and Immunity* 58:93
- 10 34. McCafferty *et al.* (1995) *Infection and Immunity* 63:2387-9.
35. Knudsen *et al.* (1996) Third Meeting of the European Society for Chlamydia Research, Vienna
36. Wiedmann-Al-Ahmad M, *et al.* Reactions of polyclonal and neutralizing anti-p54 monoclonal antibodies with an isolated, species-specific 54-kilodalton protein of *Chlamydia pneumoniae*. *Clin Diagn Lab Immunol.* 1997 Nov; 4(6): 700-704
- 15 37. Hughes *et al.*, 1992. *Infect. Immun.* 60(9):3497,
38. Dion *et al.*, 1990. *Virology* 179:474-477,
39. Snijders *et al.*, 1991. *J. Gen. Virol.* 72:557-565,
40. Langeveld *et al.*, *Vaccine* 12(15):1473-1480, 1994
41. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons Inc., 1994
- 20 42. Kunkel *et al.* *Proc. Natl. Acad. Sci. USA* (1985) 82:448
43. Silhavy *et al.* *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press, 1984
44. Davis *et al.* *A Manual for Genetic Engineering: Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory Press, 1980)
45. Casey & Davidson, *Nucl. Acid Res.* (1977) 4:1539
- 25 46. Cagnon *et al.*, *Protein Engineering* (1991) 4(7):843
47. Takase *et al.*, *J. Bact.* (1987) 169:5692

attttaacgt	gcgtatcatt	tgtgactaag	agatagactt	gctttcttta	tctatcttct	60
gtattggaaa	gaaagcccct	tgagggaaaa	aaaggttggt	atg aag att cca ctc	115	
				Met Lys Ile Pro Leu		
				1 5		
cgc ttt tta	ttg atata	caatta	gtatcct	acg ctt tct atg	163	
Arg Phe Leu	Leu Ile Ser	Leu Val Pro	Thr Leu Ser	Met Ser Asn Leu		
	10		15	20		
tta gga gct	gct act acc	gaa gag tta	tcg gct agc aat	age ttc gat	211	
Leu Gly Ala	Ala Thr Thr	Glu Glu Leu Ser	Ala Ser Asn Ser	Phe Asp		
	25	30	35			
gga act aca	tca aca aca	agc ttt tct	agt aaa aca	tca tcg gct aca	259	
Gly Thr Thr	Ser Thr Thr	Ser Phe Ser	Ser Lys Thr	Ser Ser Ala Thr		
	40	45	50			
gat ggc acc	aat tat gtt	ttt aaa gat	tct gta gtt	ata gaa aat gta	307	
Asp Gly Thr	Asn Tyr Val	Phe Lys Asp	Ser Val Val	Ile Glu Asn Val		
	55	60	65			
ccc aaa aca	ggg gaa act	cag tct act	agt tgt ttt	aaa aat gac gct	355	
Pro Lys Thr	Gly Glu Thr	Gln Ser Thr	Ser Cys Phe	Lys Asn Asp Ala		
	70	75	80	85		
gca gct gga	gat cta aat	ttc tta gga	ggg gga ttt	tct ttc aca ttt	403	
Ala Ala Gly	Asp Leu Asn	Phe Leu Gly	Gly Gly Phe	Ser Phe Thr Phe		
	90	95	100			
agc aat atc	gat gca acc	acg gct tct	gga gct gct	att gga agt gaa	451	
Ser Asn Ile	Asp Ala Thr	Thr Ala Ser	Gly Ala Ala	Ile Gly Ser Glu		
	105	110	115			
gca gct aat	aag aca agt	cac gct tta	tca gga ttt	tcg gca ctt tct	499	
Ala Ala Asn	Lys Thr Val	Thr Leu Ser	Gly Phe Ser	Ala Leu Ser Phe		
	120	125	130			
ctt aaa tcc	cca gca agt	acagtg act	aat gga ttg	ggagct atc aat	547	
Leu Lys Ser	Pro Ala Ser	Thr Val Thr	Asn Gly Leu	Gly Ala Ile Asn		
	135	140	145			
gtt aaa ggg	aat tta agc	cta ttg gat	aat gat aag	gta ttg att cag	595	
Val Lys Gly	Asn Leu Ser	Leu Leu Asp	Asn Asp Lys	Val Leu Ile Gln		
	150	155	160	165		
gac aat ttc	tca aca gga	gat ggc gga	gca att aat	tgt gca ggc tcc	643	
Asp Asn Phe	Ser Thr Gly	Asp Gly Gly	Ala Ile Asn	Cys Ala Gly Ser		
	170	175	180			
ttg aag atc	gca aac aat	aag tcc ctt	tct ttt att	gga aat agt tct	691	
Leu Lys Ile	Ala Asn Asn	Lys Ser Leu	Ser Phe Ile	Gly Asn Ser Ser		
	185	190	195			
tca aca cgt	ggc gga ggc	gata tca	acccaaaa	aaactc	739	
Ser Thr Arg	Gly Gly Ala	Ile His Thr	Lys Asn Leu	Thr Leu Ser Ser		
	200	205	210			
ggt ggg gaa	act act aat	tttcag	ggg aat	acac ggc cct	787	
Gly Gly Glu	Thr Leu Phe	Gln Gly Asn	Thr Ala Pro	Thr Ala Ala Gly		
	215	220	225			

aaa gga ggt gct atc gcg att gca gac tct ggc acc cta tcc att tct 835
 Lys Gly Gly Ala Ile Ala Ile Ala Asp Ser Gly Thr Leu Ser Ile Ser
 230 235 240 245

gga gac agt ggc gac att atc ttt gaa ggc aat acg ata gga gct aca 883
 Gly Asp Ser Gly Asp Ile Ile Phe Glu Gly Asn Thr Ile Gly Ala Thr
 250 255 260

gga acc gtc tct cat agt gct att gat tta gga act agc gct aag ata 931
 Gly Thr Val Ser His Ser Ala Ile Asp Leu Gly Thr Ser Ala Lys Ile
 265 270 275

act gcg tta cgt gct gcg caa gga cat acg ata tac ttt tat gat ccg 979
 Thr Ala Leu Arg Ala Ala Gln Gly His Thr Ile Tyr Phe Tyr Asp Pro
 280 285 290

att act gta aca gga tcg aca tct gtt gct gat gct ctc aat att aat 1027
 Ile Thr Val Thr Gly Ser Thr Ser Val Ala Asp Ala Leu Asn Ile Asn
 295 300 305

agc cct gat act gga gat aac aaa gag tat acg gga acc ata gtc ttt 1075
 Ser Pro Asp Thr Gly Asp Asn Lys Glu Tyr Thr Gly Thr Ile Val Phe
 310 315 320 325

tct gga gag aag ctc acg gag gca gaa gct aaa gat gag aag aac cgc 1123
 Ser Gly Glu Lys Leu Thr Glu Ala Glu Ala Lys Asp Glu Lys Asn Arg
 330 335 340

act tct aaa tta ctt caa aat gtt gct ttt aaa aat ggg act gta gtt 1171
 Thr Ser Lys Leu Leu Gln Asn Val Ala Phe Lys Asn Gly Thr Val Val
 345 350 355

tta aaa ggt gat gtc gtt tta agt gcg aac ggt ttc tct cag gat gca 1219
 Leu Lys Gly Asp Val Val Leu Ser Ala Asn Gly Phe Ser Gln Asp Ala
 360 365 370

aac tct aag ttg att atg gat tta ggg acg tcg ttg gtt gca aac acc 1267
 Asn Ser Lys Leu Ile Met Asp Leu Gly Thr Ser Leu Val Ala Asn Thr
 375 380 385

gaa agt atc gag tta acg aat ttg gaa att aat ata gac tct ctc agg 1315
 Glu Ser Ile Glu Leu Thr Asn Leu Glu Ile Asn Ile Asp Ser Leu Arg
 390 395 400 405

aac ggg aaa aag ata aaa ctc agt gct gcc aca gct cag aaa gat att 1363
 Asn Gly Lys Lys Ile Lys Leu Ser Ala Ala Thr Ala Gln Lys Asp Ile
 410 415 420

cgt ata gat cgt cct gtt gta ctg gca att agc gat gag agt ttt tat 1411
 Arg Ile Asp Arg Pro Val Val Leu Ala Ile Ser Asp Glu Ser Phe Tyr
 425 430 435

caa aat ggc ttt ttg aat gag gac cat tcc tat gat ggg att ctt gag 1459
 Gln Asn Gly Phe Leu Asn Glu Asp His Ser Tyr Asp Gly Ile Leu Glu
 440 445 450

tta gat gct ggg aaa gac atc gtg att tct gca gat tct cgc agt ata 1507
 Leu Asp Ala Gly Lys Asp Ile Val Ile Ser Ala Asp Ser Arg Ser Ile
 455 460 465

gat gct gta caa tct ccg tat ggc tat cag gga aag tgg acg atc aat 1555
 Asp Ala Val Gln Ser Tyr Gly Tyr Gln Gly Lys Trp Thr Ile Asn
 470 475 480 485

tgg tct act gat gat aag aaa gct acg gtt tct tgg gcg aag cag agt 1603
 Trp Ser Thr Asp Asp Lys Lys Ala Thr Val Ser Trp Ala Lys Gln Ser
 490 495 500

ttt aat ccc act gct gag cag gag gct ccg tta gtt cct aat ctt ctt 1651
 Phe Asn Pro Thr Ala Glu Gln Glu Ala Pro Leu Val Pro Asn Leu Leu
 505 510 515

tgg ggt tct ttt ata gat gtt cgt tcc ttc cag aat ttt ata gag cta 1699
 Trp Gly Ser Phe Ile Asp Val Arg Ser Phe Gln Asn Phe Ile Glu Leu
 520 525 530

ggt act gaa ggt gct cct tac gaa aag aga ttt tgg gtt gca ggc att 1747
 Gly Thr Glu Gly Ala Pro Tyr Glu Lys Arg Phe Trp Val Ala Gly Ile
 535 540 545

tcc aat gtt ttg cat agg agc ggt cgt gaa aat caa agg aaa ttc cgt 1795
 Ser Asn Val Leu His Arg Ser Gly Arg Glu Asn Gln Arg Lys Phe Arg
 550 555 560 565

cat gtg agt gga ggt gct gta gta ggt gct agc acg agg atg ccg ggt 1843
 His Val Ser Gly Gly Ala Val Val Gly Ala Ser Thr Arg Met Pro Gly
 570 575 580

ggt gat acc ttg tct ctg ggt ttt gct cag ctc ttt gcg cgt gac aaa 1891
 Gly Asp Thr Leu Ser Leu Gly Phe Ala Gln Leu Phe Ala Arg Asp Lys
 585 590 595

gac tac ttt atg aat acc aat ttc gca aag acc tac gca gga tct tta 1939
 Asp Tyr Phe Met Asn Thr Asn Phe Ala Lys Thr Tyr Ala Gly Ser Leu
 600 605 610

cgt ttg cag cac gat gct tcc cta tac tct gtg gtg agt atc ctt tta 1987
 Arg Leu Gln His Asp Ala Ser Leu Tyr Ser Val Val Ser Ile Leu Leu
 615 620 625

gga gag gga gga ctc cgc gag atc ctg ttg cct tat gtt tcc aag act 2035
 Gly Glu Gly Gly Leu Arg Glu Ile Leu Leu Pro Tyr Val Ser Lys Thr
 630 635 640 645

ctg ccg tgc tct ttc tat ggg cag ctt agc tac ggc cat acg gat cat 2083
 Leu Pro Cys Ser Phe Tyr Gly Gln Leu Ser Tyr Gly His Thr Asp His
 650 655 660

cgc atg aag acc gag tct cta ccc ccc ccc ccc ccg acg ctc tcg acg 2131
 Arg Met Lys Thr Glu Ser Leu Pro Pro Pro Pro Thr Leu Ser Thr
 665 670 675

gat cat act tct tgg gga gga tat gtc tgg gct gga gag ctg gga act 2179
 Asp His Thr Ser Trp Gly Gly Tyr Val Trp Ala Gly Glu Leu Gly Thr
 680 685 690

cga gtt gct gtt gaa aat acc agc ggc aga gga ttt ttc caa gag tac 2227
 Arg Val Ala Val Glu Asn Thr Ser Gly Arg Gly Phe Phe Gln Glu Tyr
 695 700 705

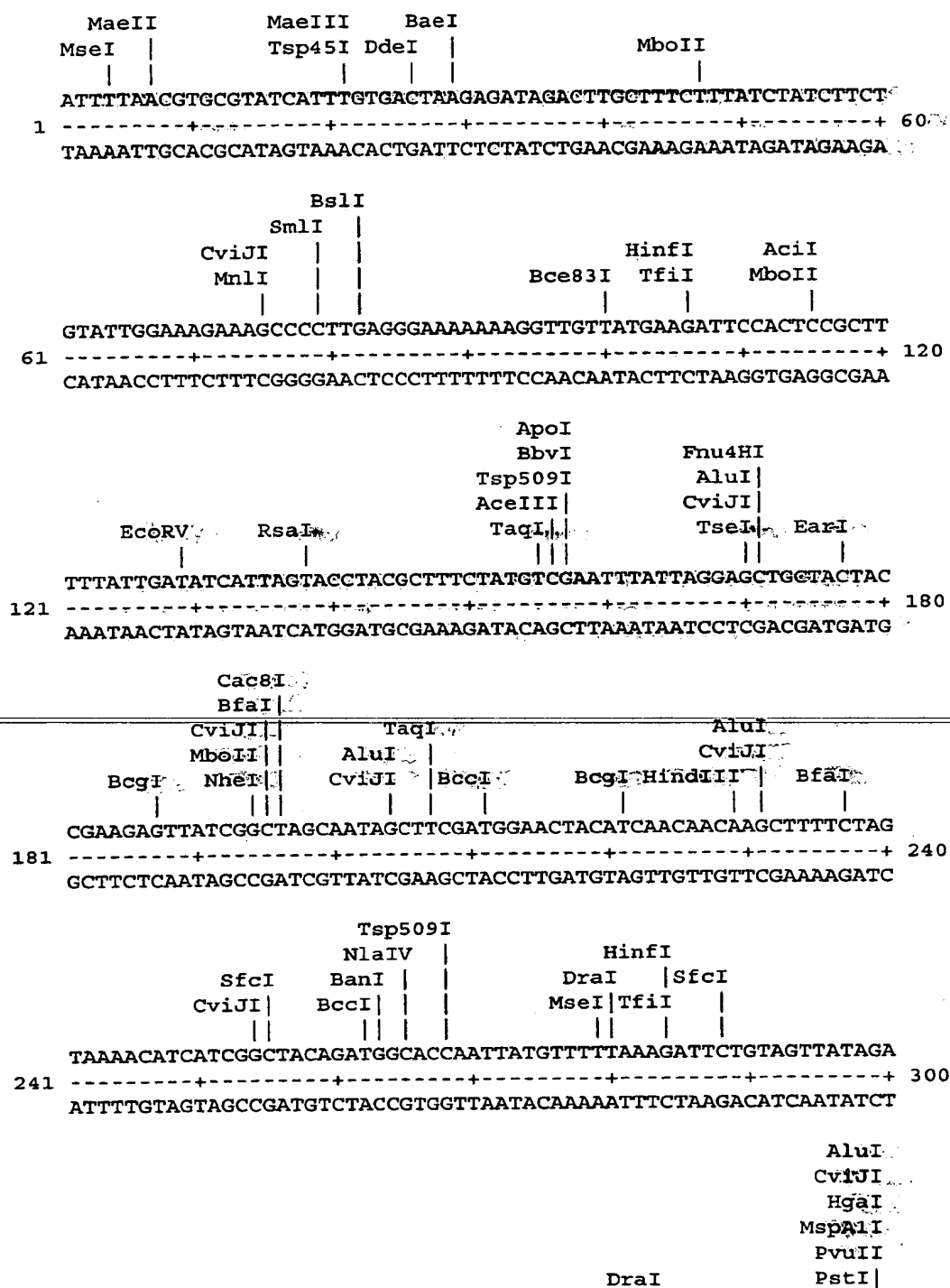
act cca ttt gta aaa gtc caa gct gtt tac gct cgc caa gat agc ttt 2275
 Thr Pro Phe Val Lys Val Gln Ala Val Tyr Ala Arg Gln Asp Ser Phe
 710 715 720 725

gta gaa cta gga gct atc agt cgt gat ttt agt gat atc gat cat ctt tat 2323
 Val Glu Leu Gly Ala Ile Ser Arg Asp Phe Ser Asp Ser His Leu Tyr
 730 735 740

35

Figure 2

Restriction Enzyme analysis of CPN100397



MseI | Fnu4HI |
 BbvI | CviRI |
 BseMII | TseI |
 BfaI | Fnu4HI |
 SpeI | SfcI |
 RsaI | BslI | DdeI | AccI | TseI |
 AAATGTACCCAAAACAGGGGAACTCAGTCTACTAGTTGTTTAAAAATGACGCTGCAGC
 301 -----+-----+-----+-----+-----+ 360
 TTACATGGGTTTTGTCCCTTTGAGTCAGATGATCAACAAAATTTTACTGCGACGTCG
 ApoI | AceIII |
 Tsp509I | BsaJI |
 BbvI | BstDSI |
 DpnI | CviRI |
 BglII | CjeI |
 BstYI | DdeI | ClaI |
 Sau3AI | MnlI | BpmI | SfaNI | TaqI |
 TGGAGATCTAAATTTCTTAGGAGGGGATTTTCTTTCACATTTAGCAATATCGATGCAAC
 361 -----+-----+-----+-----+-----+ 420
 ACCTCTAGATTTAAAGAATCCTCCCCCTAAAAGAAAGTGTAATCGTTATAGCTACGTTG
 Bcefi |
 Fnu4HI |
 AluI |
 CviJI |
 TseI |
 MwoI | AluI | Hpy178III |
 Hpy178III | CviJI | MaeII |
 CviJI | Fnu4HI | MaeIII |
 MwoI | TseI | TaaI |
 BbvI | BpmI | Tsp45I |
 CjeI | BbvI |
 CACGGCTTCTGGAGCTGCTATTGGAAGTGAAGCAGCTAATAAGACAGTCACGTTATCAGG
 421 -----+-----+-----+-----+-----+ 480
 GTGCCGAAGACCTCGACGATAACCTTCACTTCGTCGATTATTCTGTCTAGTGCAATAGTCC
 MaeIII |
 TaaI |
 Tsp45I |
 RsaI | AluI |
 MseI | TatI | TspRI | CviJI |
 ATTTTCGGCACTTTCTTTTCTTAAATCCCCAGCAAGTACAGTGACTAATGGATTGGGAGC
 481 -----+-----+-----+-----+-----+ 540
 TAAAGCCGTGAAAGAAAAGAATTTAGGGGTCGTTTCATGTCACTGATTACCTAACCTCG
 ApoI | Hpy178III |
 Tsp509I | CviJI |
 MseI | MseI |
 TATCAATGTTAAAGGGAATTTAAGCCTATTGGATAATGATAAGGTATTGATTACAGGACAA

541 -----+-----+-----+-----+-----+ 600
 ATAGTTACAATTTCCCTTAAATTTCGGATAACCTATTACTATTCCATAACTAAGTCCTGT

Tsp509I
 MseI
 VspI NlaIV
 Tsp509I CviJI
 EcoRI Cae84I DpnI
 AclI CviRI BsmBI MboII
 BclI CjeI Sau3AI BsgI

TTTCTCAACAGGAGATGGGGGAGCAATTAATTGTGCAGGCTCCTTGAAGATGGCAAGAA
 601 -----+-----+-----+-----+-----+ 660
 AAAGAGTTGTCTCTACCGCTCGTTAATTAACACGTCCGAGGAACCTTCTAGCGTTTGT

BsaAI
 PmlI
 MaeII
 XmnI AflIII EciI HinfI
 Tth111III MboII BsbI AclI TfiI

TAAGTCCCTTTCTTTTATTGGAAATAGTTCTTCAACACGTGGCGGAGCGATTTCATACCAA
 661 -----+-----+-----+-----+-----+ 720
 ATTCAGGGAAAGAAAATAACCTTTATCAAGAAGTTGTGCACCGCCTCGCTAAGTATGGTT

Fnu4HI
 CviJI
 TseI
 MwoI
 BbvI HaeIII
 CjeI HhaI

MboII MnlI
 AAACCTCACACTATCTTCTGGTGGGGAACTCTATTTCAGGGGAATACAGCGCCTACGGC
 721 -----+-----+-----+-----+-----+ 780
 TTTGGAGTGTGTATAGAGACGACCCCTTTGAGATAAAGTCCCGTTATGTGEGGGATGGCG

NlaIV
 BanI
 AlwNI
 HinfI
 CviRI
 PleI
 Hpy178III
 NruI
 Hin4I ThaI
 MnlI BceI CjeI

Hpy178III
 BsmAI
 TGCTGGTAAAGGAGGTGCTATCGCGATTGCAGACTCTGGCACCTATCCATTTCTGGAGA
 781 -----+-----+-----+-----+-----+ 840
 ACGACCATTTCCTCCACGATAGCGCTAAGCTGTGAGACCGTGGGATAGGTAAAGACCTCT

SfcI PpiI
 AluI TaaI BsmAI
 CviNI NlaIV BsmBI

TspRI
 TaaI BpmI
 CAGTGGCGACATTATCTTTGAAGCAATACGATAGGAGCTACAGGAACCGTCTCTCATAG

841 -----+-----+-----+-----+-----+ 900
 GTCACCGCTGTAATAGAACTTCCGTTATGCTATCCTCGATGTCCTTGGCAGAGAGTATC

HhaI
 FspI
 Fnu4HI
 TseI
 DdeI
 BsaAI
 HaeII
 MwoI
 HhaI
 MaeII
 Eco47III
 MaeIII
 BfaI
 BbvI

TGCTATTGATTTAGGAAGCTAGCGCTAAGATAACTGCGTTACGTGCTGCGCAAGGACATAC
 901 -----+-----+-----+-----+-----+ 960
 ACGATAACTAAATCCTTGATCGCGATTCTATTGACGCAATGCACGACGCGTTCCTGTATG

AlwI
 SfaNI
 Hpy188IX
 DpnI
 BsaBI
 Sau3AI
 MaeIII
 DpnI
 AlwI
 TaaI
 Sau3AI

GATATACTTTTATGATCCGATTACTGTAACAGGATCGACATCTGTTGCTGATGCTCTCAA
 961 -----+-----+-----+-----+-----+ 1020
 CTATATGAAAATACTAGGCTAATGACATTGTCCTAGCTGTAGACAACGACTACGAGAGTT

DrdII
 NlaIV
 BscGI
 BpmI
 MseI
 VspI
 BstZ17I
 SspI
 CviJI
 BslI
 BsrI
 Sth132I
 AccI
 Hpy178III

TATTAATAGCCCTGATACTGGAGATAACAAAGAGTATACGGGAACCATAGTCTTTTCTGG
 1021 -----+-----+-----+-----+-----+ 1080
 ATAATTATCGGGACTATGACCTCTATTGTTTCTCATATGCCCTTGGTATCAGAAAAGACC

AluI
 CviJI
 MnlI
 Hin4I
 BpmI
 AluI
 BplI
 CviJI
 Tsp509I
 AciI
 MboII

AGAGAAGCTCACGGAGGCAGAAGCTAAAGATGAGAAGAACCGCACTTCTAAATTACTTCA
 1081 -----+-----+-----+-----+-----+ 1140
 TCTCTTCGAGTGCCTCCGTCTTCGATTCTACTCTTCTTGCGTGAAGATTTAATGAAGT

BsmFI
 DraI
 HphI
 DraI
 MseI
 SfcI
 MseI
 MseI

AAATGTTGCTTTTAAAAATGGGACTGTAGTTTAAAAAGGTGATGTCGTTTAAAGTGCAGAA
 1141 -----+-----+-----+-----+-----+ 1200
 TTTACAACGAAAATTTTACCCTGACATCAAAATTTTCCACTACAGCAAAATTCACGCTT

	MunI
RsaI	Tsp509I
BsrGI	DpnI

TatI CviJI Sau3AI AccI

1501 CAGTATAGATGCTGTACAATCTCCGTATGGTATCAGGGAAAGTGGACGATCAATTGGTC
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1560
 GTCATATCTACGACATGTTAGAGGCATACCGATAGTCCCTTTACCTGCTAGTTAACCAG

MnII
 TspRI
 BseMII Bpu1102I
 MseI DdeI
 AluI CviJI TaaI Hin4I BtsI

1561 TACTGATGATAAGAAAGCTACGGTTTCTTGGGCGAAGCAGAGTTTTAATCCCACTGCTGA
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1620
 ATGACTACTATTCTTTTCGATGCCAAAGAACCCGCTTCGTCTCAAATTAGGGTGACGACT

BsaXI
 NlaIV
 CviJI MboII

1621 GCAGGAGGCTCCGTTAGTTCCTAATCTTCTTTGGGGTCTTTTATAGATGTTTCGTTCCCTT
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1680
 CGTCCTCCGAGGCAATCAAGGATTAGAAGAAACCCCAAGAAAATATCTACAAGCAAGGAA

ApoI BfaI BsiHKAI
 Tsp509I AluI Bsp1286I Eco57I CviRI
 Hpy178III CviJI RsaI

1681 CCAGAATTTTATAGAGCTAGGTACTGAAGGTGCTCCTTACGAAAAGAGATTTTGGGTTGC
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1740
 GGTCTTAAATATCTCGATCCATGACTTCCACGAGGAATGCTTTTCTCTAAAACCCAACG

Hpy178III
 BsiEI
 BsaXI MslI
 Acil ApoI MnlI
 CviRI BsrBI Tsp509I NlaIII

1741 AGGCATTTCCAATGTTTTGCATAGGAGCGGTTCGTGAAAATCAAAGGAAATTCGTCATGT
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1800
 TCCGTAAAGGTTACAAAACGTATCCTCGCCAGCACTTTTAGTTTCCTTTAAGGCAGTACA

MslI
 Sth132I
 BssSI
 Cac8I NciI HaeIV BsmAI
 CjePI ScrFI Hin4I CjePI
 BfaI MspI FokI HphI
 MnlI SfaNI SfcI BsaXI NheI

1801 GAGTGGAGGTGCTGTAGTAGGTGCTAGCACGAGGATGCCGGGTGGTGATACCTTGCTCTCT
 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1860
 CTCACCTCCACGACATCATCCACGATCGTGCTCCTACGGCCCACTATGGAACAGAGA

AceIII
 BseMII
 MaeIII
 Tsp45I
 AluI
 CviJI
 HhaI
 Bpu1102I
 DdeI
 MwoI
 Tsp509I

GGGTCTTTGCTCAGGCTCTTTGGGGCTGACAAAGACTACTTTATGAATAGCAATTTGGCAAA
 1861 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1920
 CCCAANAGGAGTCGAGAAAGCGGCACTGTTTCTGATGAAATACTTATGCTTAAAGCGTTT

Fnu4HI
 CviRI
 TseI
 DpnI
 SfaNI
 BstYI
 AlwI
 Sau3AI
 MaeII
 BbvI

GACCTACGCAGGATCTTTACGTTTGCAGCACGATGCTTCCCTATACTCTGTGGTGAGTAT
 1921 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1980
 CTGGATGCGTCCTAGAAAATGCAAACGTCGTGCTACGAAGGGATATGAGACACCACTCATA

DpnI
 BstYI
 Sau3AI
 BciVI
 MnlI
 HphI
 MnlI
 PleI
 HinfI
 AclI
 AlwI
 ThaI
 PleI
 BceI
 HinfI

CCTTTTAGGAGAGGGAGGAGCTCCGGGAGATGCTGTTGGCTTATGTTTCCAGACTCTGCC
 1981 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2040
 GGAAATCCTCTCTCTCTCTGAGGGGCTCTAGGACACCGAATAGAAAGGTTCTGAGACGG

CviJI
 HaeIII
 BbvI
 EaeI
 GdiII
 BbsI
 Hin4I
 HinfI
 Tth111I
 NlaIII
 AlwI
 BceI
 DpnI
 Sau3AI
 BsiHKA
 Bsp1286I
 Fnu4HI
 TseI
 AluI
 CviJI
 Bpu1102I
 DdeI
 AluI
 CviJI

GTGCTCTTTCTATGGGCGAGCTTAGCTAGCGCCCATACCGATCATCGCATGAGACCGAGTC
 2041 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2100
 CACGAGAAAGATACCGCTGGAATCGATGCGCGGTATGCGTAGTACCGTAGCTCTGCGCTCAG

DpnI
 Sau3AI
 HgaI
 TaqII

PleI | Hpy178III | | | AceIII
 BsmAI | | | TaqI | | | MnlI | BsaXI |
 MboII | | | Sth132I | | | AlwI | | | Hin4I |
 TCTACCCCCCCCCCCCCGACGCTCTCGACGGATCATACTTCTTGGGGAGGATATGTCTG
 2101 -----+-----+-----+-----+-----+ 2160
 AGATGGGGGGGGGGGGGGCTGCGAGAGCTGCCTAGTATGAAGAACCCTCCTATACAGAC

Fnu4HI
 | | | TauI
		AcII
		MspAI
		MnlI
CviJI	AluI	SmlI
		XhoI
 GGCTGGAGAGCTGGGAACCTCGAGTTGCTGTTGAAAATACCAGCGGCAGAGGATTTTCCCA
 2161 -----+-----+-----+-----+-----+ 2220
 CCGACCTCTCGACCCTTGAGCTCAACGACAACCTTTTATGGTCGCCGTCTCCTAAAAAGGT

RsaI | AluI | Cac8I | AluI
 | | | CviJI | MwoI | | MwoI |
 | | | | | |
 AGAGTACACTCCATTTGTAAAAGTCCAAGCTGTTTACGCTCGCCAAGATAGCTTTGTAGA
 2221 -----+-----+-----+-----+-----+ 2280
 TCTCATGTGAGGTAAACATTTTCAGGTTTCGACAAATGCGAGCGGTTCTATCGAAACATCT

BsaBI
 | | |
 AluI | HinfI | CjePI | HinfI
 BfaI CviJI Hpy178III | TfiI | | SfaNI | TfiI
 | | | | | |
 ACTAGGAGCTATCAGTCGTGATTTTAGTGATTGCGCATCTTTATAACCTTGCATTCTCT
 2281 -----+-----+-----+-----+-----+ 2340
 TGATCCTCGATAGTCAGCACTAAATCACTAAGCGTAGAAATATTGGAACGCTAAGGAGA

MnlI
 HinfI | | | CviRI | BpmI | Tth111III
 TfiI | | | | | |
 | | | CjePI TaaI | | | SspI | NlaIII | | Hin4I |
 TGGAATCAAGTTAGAGAAACGGTTTGCAGAGCAATATTATCATGTTGTAGCGATGTATTC
 2341 -----+-----+-----+-----+-----+ 2400
 ACCTTAGTTCAATCTCTTTGCCAAACGTCTCGTTATAATAGTACAACATCGCTACATAAG

BslI
 | | |
 MaeIII | BsaJI |
 Hpy178III | Pfl1108I | | RsaI | | MmeI | | StyI |
 TCCAGATGTTTGTCTAGTAACCCCAAATGTACGACTACCCTACTTTCCAACCAAGGGAG
 2401 -----+-----+-----+-----+-----+ 2460
 AGGTCTACAAACAGCATCATTGGGGTTTACATGCTGATGGGATGAAAGGTTGGTTCCTC

Bsu36I
 DdeI
 CviJI |
 HaeI |
 HaeIII |
 StuI | AceIII
 NspV
 TaqI
 MboII |
 MmeI | | CviJI
 BbsI | | DdeI | MwoI |

[illegible][illegible][illegible]

```

                                CviRI      DpnI
                              MboII       Sau3AI |
                             TspRI        Hpy188IX|
                           TaaI           AlwI---||
                          BbsI            Hin4I ||
                               ||          ||   ||
ATTTCAAAGAAGACACTGTTGCATTAGATACGGTCTCTGATCGCTCAAAA
2701 -----+-----+-----+-----+-----+-----+-----2750
TAAAGTTTCTTCTGTGACAACGTAATCTATGCGAGAGACTAGGGAGTTTT

```

Year	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100
1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	

☐ Page(s) _____ of Declarations were not present
for scanning. (Document title)

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☐ Scanned copy is best available.



1
2
3
4
5